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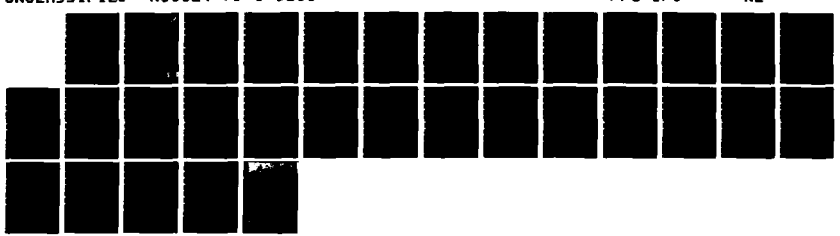

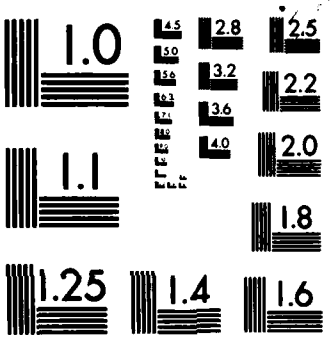

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THE COLLECTION, PURIFICATION, AND CRYOPRESERVATION OF CANINE PERIPHERAL BLOOD MONONUCLEAR CELLS OBTAINED AS A BY-PRODUCT OF PLATELET APHERESIS PROCEDURES

by

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An average of 1.69×10^9 white blood cells was collected from each of 11 Beagle dogs during 169 routine platelet apheresis procedures using a Haemonetics Model 30 Blood Cell Separator. Seventy-four percent of the collected white blood cells were mononuclear cells.

Treatment of the mononuclear cells with ficoll-hypaque to remove the granulocytes and red blood cells resulted in a loss of 25% of the mononuclear cells. After freezing with 10% DMSO, whether as buffy coats or after ficoll-hypaque treatment, thawing and washing, about 70% of the mononuclear cells were

recovered as viable cells. Recovery values of viable mononuclear cells were similar whether there was rapid addition of the cryoprotectant and freezing at 2-3 C/minute in a -80 C mechanical freezer or slow addition of the cryoprotectant and freezing at 1 C/minute in a controlled rate freezer.

SUMMARY

An average of 1.69 X 10^9 white blood cells was collected from each of 11 Beagle dogs during 169 routine platelet apheresis procedures using a Haemonetics Model 30 Blood Cell Separator. Seventy-four percent of the collected white blood cells were mononuclear cells.

Treatment of the mononuclear cells with ficoll-hypaque to remove the granulocytes and red blood cells resulted in a loss of 25% of the mononuclear cells. After freezing with 10% DMSO, whether as buffy coats or after ficoll-hypaque treatment, thawing and washing, about 70% of the mononuclear cells were recovered as viable cells. Recovery values of viable mononuclear cells were similar whether there was rapid addition of the cryoprotectant and freezing at 2-3 C/minute in a -80 C mechanical freezer or slow addition of the cryoprotectant and freezing at 1 C/minute in a controlled-rate freezer.

INTRODUCTION

Numerous investigators have demonstrated the presence of circulating stem cells in mononuclear cell concentrates isolated from the peripheral blood of man and animals.[1-4] Blood cell processors have been utilized to collect large numbers of peripheral blood mononuclear cells from humans and dogs, and the infusion of peripheral blood mononuclear cells has been shown to reconstitute bone marrow in irradiated dogs.[5-7] Recently, Lasky and coworkers have demonstrated the presence of pluripotent stem cells (CFU-GEMM) in peripheral blood mononuclear cells isolated from normal human volunteers with the Fenwal CS-3000 blood cell processor.[8] Peripheral blood mononuclear cell concentrates offer a potential alternative to bone marrow for the treatment of patients with aplastic anemia, hematologic malignancies, and solid tumors.

Graft-versus-host disease (GVHD) continues to be a problem in allogeneic transplants in spite of the advancement of transplantation immunology. Physical separation utilizing discontinuous albumin gradients and immunologic separation by treatment with monoclonal antibodies or soybean lectins and sheep erythrocytes reduces the number of immunocompetent cells in peripheral blood mononuclear cells and bone marrow and may reduce the incidence of GVHD.[9-11] Purification of the mononuclear cells by ficoll-hypaque treatment is an integral component of most current stem cell separation procedures.

Autologous transplantation in hematologic malignancies and solid tumors eliminates the problem of GVHD, but frequently the cells must be cryopreserved

until they are needed for marrow reconstitution.[12,13] Current methods of bone marrow and peripheral blood mononuclear cell cryopreservation are usually cumbersome and require controlled rate freezing at 1 C/minute.[14,15]

In the study reported here, we evaluated the number of peripheral blood mononuclear cells collected from dogs during routine platelet apheresis procedures using the Haemonetics Model 30 Blood Cell Separator. We also measured the recovery of mononuclear cells after ficoll-hypaque treatment. The effects of the rate of addition of the cryoprotectant and the freezing rate on the viability and recovery of cryopreserved canine peripheral blood mononuclear cells were assessed.

MATERIALS AND METHODS

Collection of Platelets and Mononuclear Cells by Discontinuous-Flow Centrifugation Using the Haemonetics 30 Blood Processor

Repeated cytapheresis procedures were performed in 11 healthy Beagle dogs. Two weeks before the first apheresis procedure, an arterio-venous (AV) fistula was surgically created between the carotid artery and jugular vein to provide sufficient pressure in the vein to allow adequate blood flow to the blood processing apparatus. For blood component collection, an outflow catheter (16 gauge) was inserted into the venous segment of the AV fistula. A return catheter (18 gauge) was inserted into a vein in the foreleg. A Haemonetics pediatric bowl (125 ml capacity) was used for collection, and the blood was anticoagulated with acid-citrate-dextrose (NIH, Formula A) at a ratio of 1 part ACD:7 parts whole blood. Blood was collected into the bowl at 60 ml/minute, and platelet isolation was started when the platelet-containing ring was about 2 cm from the core of the bowl. During the platelet and buffy coat collection, the flow was reduced to 20 ml/minute and was continued for either 45 or 60 seconds after red blood cells appeared in the platelet collection port. The excess plasma and red blood cells were returned to the dog. This was repeated three times, for a total of four "passes". A total of 120 to 200 ml of platelet and mononuclear cell-rich plasma was collected. Fifty to 70 ml of platelet-poor plasma was collected during one of the passes via the white blood cell collection port for use during resuspension of the platelets and mononuclear cells after freezing, thawing, and washing.

Separation of Cellular Components

The collected platelet and mononuclear cell (MNC)-rich plasma was centrifuged at 160 X g for 10 minutes in a Sorvall RC-3B refrigerated centrifuge maintained at 22 ± 2 C. The platelet-rich plasma (PRP) was expressed into a 600 ml polyvinylchloride (PVC) transfer pack with a plasma expressor (Fenwal #4R4414). The PRP was frozen, as previously described,[16] for future use. A small sample of the MNC-rich residue was obtained for determination of the number of white blood cells. White blood cell counts were determined in triplicate using the Coulter Model ZF. Smears were prepared in duplicate for differential white blood cell counts. The number of MNC's was determined by multiplying the white blood cell count by the percentage of mononuclear cells on the smears and by the volume of the residue.

After the platelets were removed, the mononuclear cells were frozen immediately as buffy coat or were purified further by ficoll-hypaque (density 1.077) density centrifugation before freezing. In 21 experiments, the residue was divided into two equal aliquots. One aliquot was transferred to a 200 ml polyolefin freezing bag (Delmed #2001) and frozen as described below. The second aliquot was purified by ficoll-hypaque density centrifugation using the following procedure. The sample was diluted with Hank's balanced salt solution (HBSS) to a concentration of approximately 2 X 10^7 leukocytes/ml. Volumes of 5 ml of diluted residue were layered over 3 ml of "Lymphoprep" solution (Nyegaard Co. A/S, Oslo, Norway) in 15 ml sterile conical centrifuge tubes using sterile technique

under a laminar flow hood. The tubes were centrifuged at 500 X g for 40 minutes at 22 ± 2 C in a Damon CRU-5000 refrigerated centrifuge. The mononuclear cell layers were harvested and pooled in a sterile 50 ml conical tube and diluted at least 1:1 with Hank's balanced salt solution. The diluted cells were mixed thoroughly and centrifuged at 500 X g for 10 minutes. The supernatants were decanted and the cells were resuspended in HBSS. The diluted cells were centrifuged again at 500 X g for 10 minutes. The supernatants were decanted and the cells were resuspended in 20 ml of cold McCoy's media. The cells were mixed thoroughly and aseptically transferred to a 200 ml polyolefin freezing bag fitted with a sampling site coupler and placed on wet ice in preparation for addition of the cryoprotectant solution.

Cryopreservation of Mononuclear Cells

A cryoprotective solution in a volume equal to that of the mononuclear cell sample to be frozen, 20 ml for purified mononuclear cells and 40 ml for buffy coat, was prepared by addition of DMSO to McCoy's medium in a ratio of 1:5. An appropriate volume of cold McCoy's medium was pipetted into a sterile bottle which was kept in ice. Pure DMSO was added to the McCoy's medium by syringe in about 1 minute with constant manual spinning of the bottle to dissipate the heat generated. With an 18 gauge, 3-1/2" spinal needle, the DMSO/McCoy's mixture was added to the cell suspension in the polyolefin freezing bag. Addition of the cryoprotectant was accomplished in one of two ways: (a) rapid addition in 1 to 2 minutes with constant agitation of the bag on ice; (b) slow addition by drip over 15 to

20 minutes with constant agitation of the bag on ice. The volume of buffy coat-cryoprotectant was approximately 80 ml; that of separated cells-cryoprotectant was about 40 ml. The final concentration of DMSO was 10% (V/V). Prior to freezing, a 0.5 ml sample was taken for total white blood cell count and white blood cell differential. The bag was placed in an aluminum container and frozen in one of two ways: (a) freezing at a rate of 2-3 C/minute by immediate horizontal placement in a -80 C mechanical freezer for 12 hours, followed by placement in the gas phase of a liquid nitrogen refrigerator (-150 C); (b) freezing in a Cryo-Med graded freezing unit at 1 C/minute from 4 C to -40 C, followed by placement in the gas phase of a liquid nitrogen refrigerator (-150 C).

Three methods to freeze the mononuclear cells were evaluated in this study: (a) rapid addition of cryoprotectant and freezing at 2-3 C/minute; (b) slow addition of cryoprotectant and freezing at 2-3 C/minute; (c) slow addition of cryoprotectant and freezing at 1 C/minute.

Thawing and Washing Procedure

The concentrates were thawed in about 30 seconds by immersion of the bag in a 42 C water bath with constant manual agitation until the last ice particle had melted. A 0.5 ml sample was immediately withdrawn aseptically by syringe and needle for white blood cell and differential white blood cell counts and for viability testing. McCoy's medium (50 ml) was added rapidly by needle through a sampling port. The diluted concentrate was transferred to a 300 ml PVC bag and centrifuged at 4160 rpm (4470 X g) for 5 minutes at 22 ± 2 C in a Sorval RC-3B centrifuge. The supernatant was drawn off through

a sterile transfer set (Fenwal #4C2244) and replaced with 35 to 50 ml of platelet-poor plasma. The cells were resuspended by gentle manual manipulation of the bag. A second 0.5 ml sample was withdrawn for testing purposes. The studies carried out after thawing and washing were: (a) total white blood cell count in triplicate using the Coulter Model ZF counter; (b) differential white blood cell counts in duplicate by staining slides with Wright's-Giemsa stain; (c) viability index using fluorescein diacetate (FDA) (green fluorescence = viable); and ethidium bromide (EB) (red fluorescence = nonviable) using a Zeiss fluorescence microscope.

In a preliminary study, a comparison was made between toluidine blue exclusion versus uptake of fluorescein diacetate and ethidium bromide[17,18] to measure the viability of peripheral blood mononuclear cells from the same sample. Nineteen fresh samples, 13 frozen-thawed and 14 frozen-thawed-washed samples were studied using both methods.

Data Analysis

Data were analyzed by the nonpaired t-test using a computer program and Hewlett-Packard 9810 computer.

RESULTS

An average of 1.69×10^9 white blood cells was collected during each of 169 platelet apheresis procedures in 11 Beagle dogs (Table 1). Seventy-four percent of the isolated white blood cells were mononuclear cells for an average of 1.2×10^9 mononuclear cells collected in the platelet apheresis residue.

When the length of platelet collection into the red blood cells was increased from 45 to 60 seconds (Table 2), a significantly greater number of white blood cells was collected. This was due to an increase in granulocytes; the number of isolated mononuclear cells was not increased by prolonging the collection time.

The white blood cells collected in the platelet apheresis residues routinely contained about 25% granulocytes and large numbers of red blood cells. Ficoll-hypaque density centrifugation removed the contaminating granulocytes and red blood cells. Approximately 25% of the mononuclear cells were lost during the purification procedure (Table 3). When one-half of the apheresis residue was treated with ficoll-hypaque centrifugation, $78 \pm 10\%$ of the mononuclear cells were recovered. When the entire apheresis residue was treated with ficoll-hypaque, $74.4 \pm 14\%$ of the mononuclear cells were recovered. The purified mononuclear cells contained less than 2% contaminating granulocytes and were virtually free of red blood cells.

A preliminary study was done to determine whether the fluorescein diacetate/ethidium bromide viability test or the toluidine blue dye exclusion test was the more sensitive method of detecting cellular damage. Because

the toluidine dye exclusion test appeared to significantly overestimate the viability of fresh, frozen-thawed, and frozen-thawed-washed mononuclear cells (Table 4), we chose the fluorescein diacetate/ethidium bromide viability test for our cryopreservation studies.

Initially, we evaluated the effect of the rate of addition of the cryoprotectant and the rate of freezing from measurements of freeze-thaw-wash recovery of viable mononuclear cells frozen as buffy coats. Buffy coat to which DMSO/McCoy's mixture was added rapidly and which was frozen at 2-3 C/minute in a -80 C mechanical freezer had viable freeze-thaw-wash recovery values similar to those of cells to which the cryoprotectant was added slowly and which were frozen at either 2-3 C/minute or at 1 C/minute in a controlled rate freezing apparatus (Table 5). When the cryoprotectant was added slowly, slightly greater ($p < 0.05$) viable freeze-thaw-wash recoveries were observed when the freezing rate was 1 C/minute. The percentage of viable mononuclear cells was always lower after thawing than that after washing and resuspension in plasma (Tables 5 and 6). There are two possible explanations for this: 1) high concentrations (10%) of DMSO in the thawed sample may have interfered with fluorescein diacetate/ethidium bromide viability testing, or 2) some cells may experience reversible damage during freezing and thawing and regain their viability after washing and resuspension in plasma.

In subsequent dog studies, we compared the cryopreservation methods used for the buffy coat and the ficoll-hypaque treated mononuclear cells. The white blood cell residue from a plateletpheresis procedure was divided into two equal aliquots. One of the aliquots was frozen as a buffy coat by one of

the three previously described cryopreservation methods. The other aliquot, [illegible] cells using the same method as its paired buffy coat. Based on the number of cells frozen, the percent of viable MNC recovered after thawing and washing was similar for both buffy coat and ficoll-hypaque treated MNC using the three cryopreservation methods (Tables 7, 8 and 9). Based on the number of cells collected, the values were significantly lower for ficoll-hypaque treated MNC than for MNC in buffy coat with all three freezing methods because of the loss of MNC during the ficoll-hypaque treatment. The differences in viable freeze-thaw-wash recovery values of ficoll-hypaque treated MNC associated with the freezing method were not significant (Table 10).

DISCUSSION

Single donor platelets obtained by apheresis with the Haemonetics Model 30 Blood Cell Separator are contaminated with large numbers of white blood cells and erythrocytes which are usually removed by centrifugation prior to transfusion of the platelets.[19] The mononuclear cell-rich residues are routinely discarded but are a potential source of pluripotent stem cells and mononuclear cells. Hunt et al have isolated monocytes from human plateletpheresis residues and cryopreserved them.[20] These cellular residues represent a source of universal donor stem cells, providing methods to remove immunocompetent cells are developed.

In our studies in dogs, we have demonstrated that there are greater than 1 X 10^9 mononuclear cells in the average plateletpheresis residue. Purification of the mononuclear cells by ficoll-hypaque density centrifugation eliminates the granulocytes and red blood cells but results in a loss of about 25% of the mononuclear cells.

The recovery of viable mononuclear cells after freezing, thawing, and washing is similar whether they are frozen as buffy coats or after ficoll-hypaque purification. Since 25% of the mononuclear cells are lost during the purification process, additional apheresis residues would be required to have an equivalent number of viable mononuclear cells.

Variations in the rate of addition of DMSO or in the freezing rate did not adversely affect the recovery of viable mononuclear cells. Freezing the cells at 2-3 C/minute in a -80 C mechanical freezer simplifies the freezing process and eliminates the need for a controlled rate freezer. Platelets

frozen with DMSO at 2-3 C/minute in a -80 C mechanical freezer have been demonstrated to have freeze-thaw-wash recoveries and in vivo recoveries equivalent to those of platelets frozen at 1 C/minute in a controlled rate freezer.[21] McGann et al have demonstrated that peripheral blood stem cells obtained from patients with chronic granulocytic leukemia have equivalent recovery values of cells and colony forming units in tissue culture (CFU-C) whether they are frozen at 1 C, 2 C, or 3 C/minute.[22] Freezing rates of this magnitude are easily achieved in a -80 C mechanical freezer by controlling the volume of cells frozen and the geometry of the freezing bag.

An advantage of freezing mononuclear cells separated from platelet apheresis collections is that the platelets can be frozen and used to support the treated patients during periods of thrombocytopenia. The cryopreserved platelets will be especially beneficial when autologous transplantations are performed.[23]

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TABLE 1

THE NUMBER OF WHITE BLOOD CELLS AND MONONUCLEAR CELLS COLLECTED FROM 11 BEAGLE DOGS DURING 169 PLATELET APHERESIS PROCEDURES USING THE HAEMONETICS MODEL 30 BLOOD PROCESSOR

	TOTAL WBC COLLECTED (X 10^9)	% MNC	TOTAL MONONUCLEAR CELLS COLLECTED (X 10^9)
MEAN	1.69	74	1.20
SD	0.50	9	0.33
RANGE	0.50-3.41	39-98	0.42-2.38

TABLE 2

THE EFFECT OF THE LENGTH OF COLLECTION INTO THE BUFFY COAT DURING PLATELET APHERESIS ON THE NUMBER OF WHITE BLOOD CELLS AND MONONUCLEAR CELLS ISOLATED USING THE HAEMONETICS MODEL 30 BLOOD PROCESSOR (MEAN ± SD)

DURATION OF COLLECTION	NUMBER OF EXPERIMENTS	TOTAL WBC ($X\ 10^9$)	% MNC	TOTAL MNC ($X\ 10^9$)
45 seconds	169	1.69 ± 0.50	74 ± 9	1.20 ± 0.33
60 seconds	13	2.03 ± 0.34	60 ± 16	1.24 ± 0.34
Significance*		$p < 0.02$	$p < 0.002$	$p > 0.5$

*Measured by nonpaired Student T-Test

TABLE 3

RECOVERY OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PLATELET APHERESIS RESIDUES AFTER SEPARATION BY FICOLL-HYPAQUE DENSITY CENTRIFUGATION.
A. 50% OF THE CELLS WERE STUDIED; B. ALL THE CELLS WERE STUDIED

A.	Number of MNC Collected ($X\ 10^9$)*	Number of MNC Remaining After Separation ($X\ 10^9$)	% Remaining After Separation
MEAN	0.66	0.51	78.0
SD	0.18	0.12	10.0
RANGE	0.40-1.19	0.28-0.78	62-100
n	19	19	19

B.	Number of MNC Collected ($X\ 10^9$)*	Number of MNC Remaining After Separation ($X\ 10^9$)	% Remaining After Separation
MEAN	1.21	0.90	74.4
SD	0.34	0.27	14.0
RANGE	0.42-2.38	0.32-1.61	37-100
n	131	131	131

TABLE 4

COMPARISON OF TOLUIDINE BLUE (TB) EXCLUSION WITH FLUORESCEIN DIACETATE (FDA/ETHIDIUM BROMIDE (EB) UPTAKE FOR MEASURING VIABILITY OF PERIPHERAL BLOOD MONONUCLEAR CELLS (MEAN ± SD)

	FRESH		FROZEN-THAWED		FROZEN-THAWED-WASHED	
	FDA/EB	TB	FDA/EB	TB	FDA/EB	TB
NUMBER OF EXPERIMENTS	19	19	13	13	14	14
MEAN % OF VIABLE CELLS	97.5	99.7	55	77	69	85
SD	2	0.6	18	17	13	9
RANGE	92-100	98.5-100	26.5-80	50-95	49.5-93	66.5-99
SIGNIFICANCE*	$p < 0.002$		$p < 0.01$		$p < 0.002$	

*Measured by nonpaired Student T-test

TABLE 5

RECOVERY OF VIABLE PERIPHERAL BLOOD MONONUCLEAR CELLS FROM DOG BUFFY COATS AFTER FREEZING, THAWING AND WASHING (MEAN ± SD)

RATE OF ADDITION OF CRYOPROTECTANT	RAPID	SLOW	SLOW
RATE OF FREEZING (C/MINUTE)	2-3	2-3	1
NUMBER OF EXPERIMENTS	8	6	7
NUMBER OF CELLS FROZEN ($X\ 10^9$)	.74 ± .44	.69 ± .14	.70 ± .10
NUMBER OF VIABLE MNC POSTTHAW ($X\ 10^9$)	.41 ± .15	.32 ± .06	.34 ± .21
% VIABLE POSTTHAW RECOVERY	56 ± 22	46 ± 11	48 ± 22
NUMBER OF VIABLE MNC POSTWASH ($X\ 10^9$)	.50 ± .23	.39 ± .11	.51 ± .20
% VIABLE FREEZE-THAW-WASH RECOVERY*	67 ± 20	57 ± 8	73 ± 16

*Rapid 2-3 C/minute versus slow 2-3 C/minute - not significant, $p > 0.5$
Slow 2-3 C/minute versus slow 1 C/minute - significant, $p < 0.05$
Rapid 2-3 C/minute versus slow 1 C/minute - not significant, $p > 0.8$

TABLE 6

VIABILITY OF MONONUCLEAR CELLS FOLLOWING FREEZING, THAWING AND WASHING AS DETERMINED BY FLUORESCEIN DIACETATE (FDA)/ETHIDIUM BROMIDE (EB) STAINING (MEAN ± SD)

RATE OF ADDITION	RATE OF FREEZING	NUMBER OF EXPERIMENTS	% VIABILITY AFTER THAWING	% VIABILITY AFTER WASHING
RAPID	2-3 C/MINUTE	8	62 ± 13	80 ± 9
SLOW	2-3 C/MINUTE	6	56 ± 16	72 ± 11
SLOW	1 C/MINUTE	7	47 ± 19	74 ± 8

TABLE 7

RECOVERY OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM 8 DOG MNC SAMPLES AFTER FREEZING AT 2-3 C/MINUTE AFTER RAPID ADDITION OF A CRYOPROTECTIVE AGENT, THAWING AND WASHING (MEAN ± SD)

	BUFFY COAT	FICOLL-HYPAQUE TREATED BUFFY COAT	
MNC COLLECTED (X 10^9)	.61 ± .24	.61 ± .24	
MNC FROZEN (X 10^9)	.61 ± .24	.46 ± .15*	
MNC VIABLE POSTTHAW (X 10^9)	.34 ± .11	.17 ± .04	
% VIABLE MNC POSTTHAW RECOVERY	56 ± 22	38 ± 8	
NUMBER MNC VIABLE POSTWASH (X 10^9)	.45 ± .22	.32 ± .09	
% VIABLE MNC POSTWASH:			
OF NUMBER FROZEN	74 ± 20	70 ± 15	($p > 0.5$)
OF NUMBER COLLECTED	74 ± 20	52 ± 14	($p < 0.05$)

*The number remaining after ficoll-hypaque treatment

TABLE 8

RECOVERY OF VIABLE PERIPHERAL BLOOD MONONUCLEAR CELLS FROM 6 DOG MNC SAMPLES AFTER FREEZING AT 2-3 C/MINUTE WITH SLOW ADDITION OF CRYOPROTECTIVE AGENT, THAWING AND WASHING (MEAN ± SD)

	BUFFY COAT	FICOLL-HYPAQUE TREATED BUFFY COAT	
MNC COLLECTED (X 10^9)	.69 ± .14	.69 ± .14	
MNC FROZEN (X 10^9)	.69 ± .14	.42 ± .17*	
MNC VIABLE POSTTHAW (X 10^9)	.31 ± .06	.23 ± .09	
% VIABLE POSTTHAW RECOVERY	46 ± 11	57 ± 11	
NUMBER OF MNC VIABLE POSTWASH (X 10^9)	.37 ± .11	.25 ± .11	
% VIABLE MNC POSTWASH:			
OF NUMBER FROZEN	54 ± 13	60 ± 9	($p > 0.2$)
OF NUMBER COLLECTED	54 ± 13	36 ± 4	($p < 0.01$)

*The number remaining after ficoll-hypaque treatment

TABLE 9

RECOVERY OF VIABLE PERIPHERAL BLOOD MONONUCLEAR CELLS FROM 7 DOG MNC SAMPLES AFTER FREEZING AT 1 C/MINUTE WITH SLOW ADDITION OF CRYOPROTECTIVE AGENT, THAWING AND WASHING (MEAN ± SD)

	BUFFY COAT	FICOLL-HYPAQUE TREATED BUFFY COAT	
MNC COLLECTED (X 10^9)	.70 ± .08	.70 ± .08	
MNC FROZEN (X 10^9)	.70 ± .08	.55 ± .08*	
MNC VIABLE POSTTHAW (X 10^9)	.34 ± .15	.32 ± .13	
% VIABLE POSTTHAW RECOVERY	48 ± 22	58 ± 22	
NUMBER OF MNC VIABLE POSTWASH (X 10^9)	.51 ± .20	.40 ± .11	
% VIABLE MNC POSTWASH:			
OF NUMBER FROZEN	73 ± 16	73 ± 12	($p > 0.8$)
OF NUMBER COLLECTED	73 ± 16	57 ± 7	($p < 0.05$)

*The number remaining after ficoll-hypaque treatment

TABLE 10

RECOVERY OF VIABLE PERIPHERAL BLOOD MONONUCLEAR CELLS FROM DOG FICOLL-HYPAQUE TREATED CELLS AFTER FREEZING, THAWING AND WASHING (MEAN ± SD)

RATE OF ADDITION OF CRYOPROTECTANT	RAPID	SLOW	SLOW
RATE OF FREEZING (C/MINUTE)	2-3	2-3	1
NUMBER OF EXPERIMENTS	8	6	7
NUMBER OF CELLS FROZEN ($X\ 10^9$)	.46 ± .15	.42 ± .17	.55 ± .08
NUMBER OF VIABLE MNC POSTTHAW ($X\ 10^9$)	.17 ± .04	.23 ± .09	.32 ± .13
% VIABLE POSTTHAW RECOVERY	38 ± 8	57 ± 11	58 ± 22
NUMBER OF VIABLE MNC POSTWASH ($X\ 10^9$)	.32 ± .09	.25 ± .11	.40 ± .11
% VIABLE FREEZE-THAW-WASH RECOVERY*	70 ± 15	60 ± 9	73 ± 12

*Rapid, 2-3 C/minute versus slow, 2-3 C/minute - $p > 0.1$
Slow, 2-3 C/minute versus slow, 1 C/minute - $p > 0.05$
Rapid, 2-3 C/minute versus slow, 1 C/minute - $p > 0.5$